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Nitrosative stress and cytokines are linked with the severity of sepsis and organ dysfunction

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ABSTRACT

Objective: An imbalance in oxidant-antioxidant status may impact the severity of sepsis. We hypothesised links between nitrosative stress and pro-inflammatory cytokines and their correlation with the severity of sepsis and associated organ dysfunction.

Methods: The hypothesis was tested in 110 patients with sepsis (in whom a disease severity score (APACHE II) and assessment of organ failure score (SOFA) were determined) and 55 healthy volunteers. Neutrophil inducible nitric oxide synthase (iNOS) expressions at mRNA and protein levels were estimated by real-time PCR and immuno-precipitation followed by Western blotting, respectively. Nitric oxide (NO) content was assessed in neutrophils by confocal microscopy, plasma nitrite by the Griess reaction and inflammatory cytokines (TNF-α, IFN-γ and IL-8) by ELISA (in plasma) and real-time PCR (in neutrophils). Serum bilirubin and creatinine were determined by routine methods and lung function by the PaO_2/FiO_2 ratio.

Results: Increased neutrophil iNOS expression and NO content, plasma total nitrite content and pro-inflammatory cytokines were present in sepsis patients (all $P < 0.001$). Plasma nitrite correlated with cytokines, APACHE II, SOFA, PaO_2/FiO_2 ratio, serum bilirubin and creatinine clearance (all r^2 0.63–0.85, $P < 0.001$). Cytokines correlated with nitrite, APACHE II, SOFA, PaO_2/FiO_2 ratio, serum bilirubin and creatinine clearance (all r^2 0.35–0.85, $P < 0.001$).

Conclusion: Neutrophils iNOS expression, NO content, plasma nitrite and cytokines have a role in the assessment of the severity of sepsis and organ toxicity.

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KEYWORDS
iNOS; NO; nitrite; pro-inflammatory cytokines; sepsis; organ dysfunction

Introduction

Sepsis is a complex clinical syndrome of host inflammatory hyper-response to an infection, whose incidence has increased over the last few decades and which is one of the most common causes of death in hospitals worldwide [1]. The multifaceted pathophysiology is characterised by the release of inflammatory mediators (e.g. neutrophil-derived cytokines) in response to an infection or inflammation. Hyper-reactivity to a systemic infection, exceeding that of a local inflammation, generates a cytokine storm often with severe pathological consequences for a number of organs and organ systems [2]. The excess production of inflammatory mediators elicits the generation of reactive oxygen and nitrogen species, including superoxide anion (O_2^-) and nitric oxide (NO), causing tissue damage and an amplified inflammatory reaction [3].

Neutrophils are the first cells recruited to the site of infection/sepsis. NO is an effector of the innate immune system where resting immune cells lack the inducible NO synthase (iNOS), a significant contributor to inflammation as it synthesises NO [4]. Various stimuli can activate different signalling pathways to initiate the expression of iNOS, such as lipopolysaccharide (LPS). In brief, LPS can bind to LPS-binding protein (LBP), which delivers LPS to CD14 [5]. Toll-like receptor-4 interacts with the CD14-LPS complex and activates signalling pathways such as those of mitogen-activated protein kinase and nuclear factor κB [6,7]. Severity of sepsis is related to bacterial endotoxin and/or levels of cytokines, which causes amplified NO production and thus increased plasma levels of total nitrite (NO_2^-). Increased NO in sepsis is linked to the induction of iNOS in macrophages and tissues, facilitated through the communications of inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and interleukin-8 (IL-8) [8].

Kidney dysfunction is common in sepsis, with diminished renal clearance of NO and total nitrite. Cytotoxic effects of NO have been reported *in vitro*; it can cause nuclear and endothelial damage, whilst red cells are also prime targets of free radical toxicity. High plasma level of total nitrite could lead to cytotoxicity or endothelial damage, resulting in alteration in vascular resistance and shock. Endotoxin increases iNOS activity, which in turn increases the release of NO by the endothelium [9]. The role of NO in

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cytokine-induced vasodilatation and resistance to vasopression has been demonstrated in patients with sepsis [10].

We previously evaluated levels of myeloperoxidase and inflammatory cytokines (TNF-α, IL-8 and IFN-γ) in the plasma of control and sepsis patients and correlated these with clinical scores (SOFA, APACHE II) and organ toxicity [11]. In the present study, we tested the hypothesis that iNOS expression, NO content, total nitrite and inflammatory cytokines (TNF-α, IFN-γ and IL-8) are linked to the severity score of sepsis and organ dysfunction.

Materials and methods

We recruited 110 critical patients admitted in the ICU having symptoms of sepsis (case group) and 55 healthy relatives accompanying the patients (control group). The study was performed in accordance with the guidelines of the ethical committees of SMS medical college and attached hospitals and Amity University Rajasthan (Reference number AIB/AUR/4761), Jaipur, India. All participants provided written informed consent. All the patients included in this study were managed by following the Surviving Sepsis Guidelines [12]. The inclusion criteria were patients having clinical evidence of infection, heart rate >100/min, respiratory rate >30/min and temperature >38 °C or <35 °C. Exclusion criteria were age >75 years, cardiac dysfunction (NYHA class III or IV), positive for hepatitis B surface antigen (HBsAg) and human immunodeficiency virus (HIV), and cancer. Clinical and demographic characteristics, including PaO_2/FiO_2 ratio (reflecting oxygen transfer to the blood, and so lung function), respiratory rate, heart rate, mean atrial pressure, SOFA and the APACHE II score, were documented for each patient separately at the time of admission in the ICU.

Venous blood was taken from controls and from patients at the time of admission to ICU for full blood count, for routine biochemistry (bilirubin, creatinine, glucose, pH, bicarbonate and procalcitonin) and for neutrophil isolation on a Percoll density gradient [11]. The latter were 95% pure (CD15, FACS-Calibur, Becton–Dickinson, San Jose, CA, USA) and 97% viable (trypan blue exclusion).

For iNOS and actin, total RNA isolation was performed using Tri reagent (Sigma, USA) in control and sepsis neutrophils. First, 5 µg of total RNA was reverse transcribed with a RevertAid H Minus First Strand cDNA Synthesis Kit using oligo (dT) primer as described by the manufacturer (Thermo Scientific, Madison, WI, USA). The cDNA was amplified by using primers for *iNOS* (F-5′ TGTGCTCTTTGCCTGTATGC3′, R 5′TTGCCAAACGTACTG GTCAC3′) and *β-actin* (F-5′AACTGGAACGGTGAAGGTG3′, R-5′CTGTGTGGACTTGGGAGAGG3′), which amplified 222 bp and 210 bp products, respectively. Quantification of iNOS mRNA by real-time PCR was carried out using the Light Cycler instrument (Bio-Rad, USA) with 2X maxima SYBR green RT-PCR master mix and the same primer and cDNA as described above. After PCR, for melting curve analysis, PCR amplicons were kept at 70 °C for 10 s and melted by raising the temperature by 0.1 °C per second up to 90 °C. Melting curve analysis consisting of one cycle: 95 °C for 15 s, 70 °C for 15 s, 95 °C for 10 s, cooling one cycle: 40 °C for 3 min was performed to demonstrate the specificity of the PCR product as a single peak. Specificity of PCR products obtained was characterised by melting curve analysis. A control, which contained all the reaction components except for the template, was included in the experiment. *β-actin* was used as the reference gene for normalisation. The differences in the quantification cycle (Cq) value for *β-actin* and *iNOS* isoform were used to calculate the expression level of iNOS.

For cytokines, total cellular RNA from neutrophils was isolated with Trizol reagent (Invitrogen Ltd., Paisley, UK) according to the manufacturer's instructions. First, 11 µL of total RNA was digested with RNase-free DNase and reverse transcribed into cDNA using the RevertAidTM H minus First Strand cDNA synthesis kit using oligo (dT) primers as per the manufacturer's instruction. cDNA were amplified with PCR (Bio-Rad Lab, Hercules, CA). The primers used were *TNF-α* (F-5′CGAGTGACA AGCCTGTAGCC3′, R-5′TTGAAGAGGACCTGGGAGTAG3′), *IFN-γ* (F-5′GCGCAAAGCCATAAATGAAC3′, R-5′CTCAGA AAGCGGAAGAGAAG3′) and *IL-8* (F-5′GCCAAGGAGTG CTAAAGA3′, R-5′CTTCTCCACAACCCTCTG3′). RT-PCR amplifications were carried out in a 96-well plate in a 25 µL reaction volume that contained 12.5 µL SYBR® Green master Mix, 1 µL of cDNA template and 0.2 mmol/L primers that were designed to amplify a part of each gene. The three-step PCR protocol applied consisted of 35 cycles of denaturation for 15 s at 95 °C and an annealing/extension step of 30 s at 57 °C for *TNF-α, IFN-γ* and *IL-8*. After PCR, for melting curve analysis, PCR amplicons were kept at 70 °C for 10 s and melted by raising the temperature by 0.1 °C per second up to 90 °C, performed to demonstrate the specificity of the PCR product as a single peak.

Control and sepsis neutrophils (total protein 750 µg) were lysed in ice-cold radio immunoprecipitation (IP) assay buffer [PBS containing 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 µg/mL aprotinin, 100 µg/mL PMSF, 20 µg/mL pepstatin, 5 mM DFP (diisopropyl fluorophosphate), 1% Triton-X 100 and 0.1% SDS] at 40°C for 30 min. Control and sepsis neutrophils (1×10^6 cells) were loaded with 10 µM 4,5-diaminofluorescein diacetate and seeded on poly-L-lysine-coated cover-slips. NO generation was monitored using a Nikon confocal microscope (A1Rsi, Tokyo, Japan) with a ×60 oil objective (PlanApo, NA1.4). Data was captured with NIS element software.

The nitrite/nitrate (NO_2^-/NO_3^-) content, indicative of NO production, was monitored by the Griess reaction. First, 100 µL of plasma was mixed with 1 µL of Nicotinamide Adenine Dinucleotide Phosphate

(NADPH) oxidase, 1 µL of 0.5 nM Flavin Adenine Dinucleotide (FAD) and 4 µL of enzyme nitrate reductase (5 U/mL dissolved in 50 mM phosphate buffer, (pH7.5); after 90 min of incubation, samples were added with 5 µL of 300 µM n-ethyl maleimide). After 2 min, the samples were diluted with 400 µL of 62.5% ethanol solution, 1:1, and reacted with Griess reagent (consisting of 1% sulfanilamide in 5% phosphoric acid and 0.1% *N*-[1-napthyl] ethylene diamine dihydrochloride). Finally, the reaction volume was treated with trichloroacetic acid, and change in absorbance was monitored by a spectrophotometer at 545 nm. Plasma TNF-α, IFN-γ and IL-8 were measured by ELISA as per the manufacturer's instructions (BD OptEIA, USA).

Data analysis was performed using SPSS v 20.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism (version 6.0; GraphPad Software, La Jolla California, USA) for windows. Data are presented as mean and standard deviation. Pearson correlation coefficient was applied to find the correlation among continuous variables. A P value <0.05 indicated statistical significance.

Results

Demographics, haematological and biochemical characteristics of participants are listed in Table 1. Cases and control were age and sex matched, but (unsurprisingly) almost all other indices were altered in the cases. In the cases, the PaO_2/FiO_2 ratio was 307.6 [5.1], the creatinine clearance was 0.27 [0.08] mL/min/kg and the SOFA and APACHE II scores were 10.0 [1.6] and 24.4 [4.2], respectively.

Transcript for *iNOS* was performed by RT-PCR, with markedly increased expression (compared to actin) in neutrophils from patients with sepsis of (6.3 [0.78]) compared to controls (1.2 [0.67]) ($P < 0.001$) (Figure 1(a)).

Table 1. Demographic, haematological and biochemical characteristics of study participants.

	Control (n = 55)	Sepsis (n = 110)	*P* Value
Age (Years)	42 ± 12	45 ± 20	0.132
Male/female ratio	32/23	61/49	0.066
Temperature (°C)	37.05 ± 0.05	38.91 ± 0.08	0.050
Heart rate (beats/min)	80 ± 1	115 ± 1	<0.001
Respiratory rate (breaths/min)	20 ± 1	29 ± 1	<0.001
MAP (mm Hg)	77 ± 8	88 ± 3	<0.001
WBC Count ($10^3/mm^3$)	6.8 ± 0.65	16.1 ± 8.02	<0.001
Haemoglobin (g/dL)	127 ± 29	97 ± 37	0.031
Platelets ($10^6/mm^3$)	260 ± 17	140 ± 52	0.001
RBS (mmol/L)	3.23 ± 2.2	6.7 ± 1.71	0.0661
Total Bilirubin (µmol/L)	19.1 ± 3.4	30.1 ± 5.6	<0.001
pH	7.15 ± 0.05	7.39 ± 0.14	<0.001
HCO_3 (mEq/L)	21.5 ± 0.43	29.3 ± 0.34	<0.001
TNF-α (pg/mL)	5.5 ± 0.7	160.7 ± 30.6	<0.001
IFN-γ (pg/mL)	4.3 ± 0.2	144.6 ± 13.4	<0.001
IL-8 (pg/mL)	3.7 ± 0.5	138 ± 7.0	<0.001
Procalcitonin (ng/mL)	0.1 ± 0.02	0.94 ± 0.04	<0.001
Neutrophil count (%)	66 ± 1	83.5 ± 1.5	<0.001
Creatinine (µmol/L)	135 ± 6	199 ± 24	<0.001

Data mean with SD. MAP, Mean Arterial Pressure; WBC, White Blood Cells; RBS, Random Blood Sugar; HCO_3, Bicarbonate.

Expression of iNOS protein was determined by IP and Western blotting (IB). iNOS (130 kDa) was present in both control and sepsis neutrophils, but, compared to actin, was markedly increased in sepsis neutrophils (6.3 [0.23] versus controls (1.2 [0.14]) ($P < 0.01$) (Figure 1(b)). Enhanced NO generation (marked by green fluorescence) in sepsis neutrophils as compared to control neutrophils is shown in Figure 1(c). Mean [SD] plasma levels of the stable metabolite of NO, total nitrite, were markedly increased in sepsis patients as compared to healthy controls (44.2 [0.25] µmol/L versus 7.2 [0.1] µmol/L, $P < 0.001$) (Figure 1(d)).

Expressions of the inflammatory cytokine genes compared to actin were determined using RT-PCR. In cases and controls, these were TNF-α 1150 [74.1] versus 24 [2.2], IFN-γ 1110 [61/7] versus 19 [1.72] and IL-8 930 [57.6] versus 12 [0.98], respectively (all $P < 0.001$) (Figure 2). Total nitrite correlated significantly with the SOFA ($r^2 = 0.71$) and APACHE II ($r^2 = 0.75$) scores, TNF-α ($r^2 = 0.85$), IFN-γ ($r^2 = 0.64$), IL-8 ($r^2 = 0.72$), the PaO_2/FiO_2 ratio ($r^2 = -0.63$), total bilirubin ($r^2 = 0.68$) and creatinine clearance ($r^2 = -0.71$) (all $P < 0.001$). Correlations between the cytokines and total nitrite and the markers of disease severity are shown in Table 2. All were highly significant: the mean correlation coefficients were TNF-α 0.70, IFN-γ 0.50 and IL-8 0.62 (ANOVA $P = 0.007$, Tukey's test TNF-α – IFN-γ $P < 0.05$), suggesting TNF-α is the preferred marker.

Discussion

Neutrophils recruited to sites of infection/inflammation release free radicals and bacteriocidal proteins, resulting in oxidative and nitrosative stress: increased levels of nitrite and cytokines are often observed at an inflammatory site [13]. NO release from neutrophils is an acknowledged moderator of microbial activity and regulator of T cell function, whilst activation of T-cells by Ca^{+2}-dependent NO generation can lead to ROS formation [14,15]. NO liberated from iNOS rapidly achieves a critical concentration for cytotoxicity and may have clinical consequences, such as in hypertension [16]. High iNOS expression has been observed in other inflammatory conditions such as acute pancreatitis and pulmonary tuberculosis [17,18].

In the present study, we hypothesised that nitrosative stress (iNOS expression/NO content/total nitrite) and pro-inflammatory cytokines (TNF-α, IFN-γ and IL-8) correlate with severity of sepsis. Our PCR analysis and IP studies showed a higher expression of iNOS in neutrophil from cases compared to controls. Our demonstration of NO generation within neutrophils provides further insights into the role of these cells in sepsis. In this context, the present multiparametric study is the primary footstep signifying comparative iNOS activity at the transcript and protein levels by means of RT-PCR and IP, respectively.

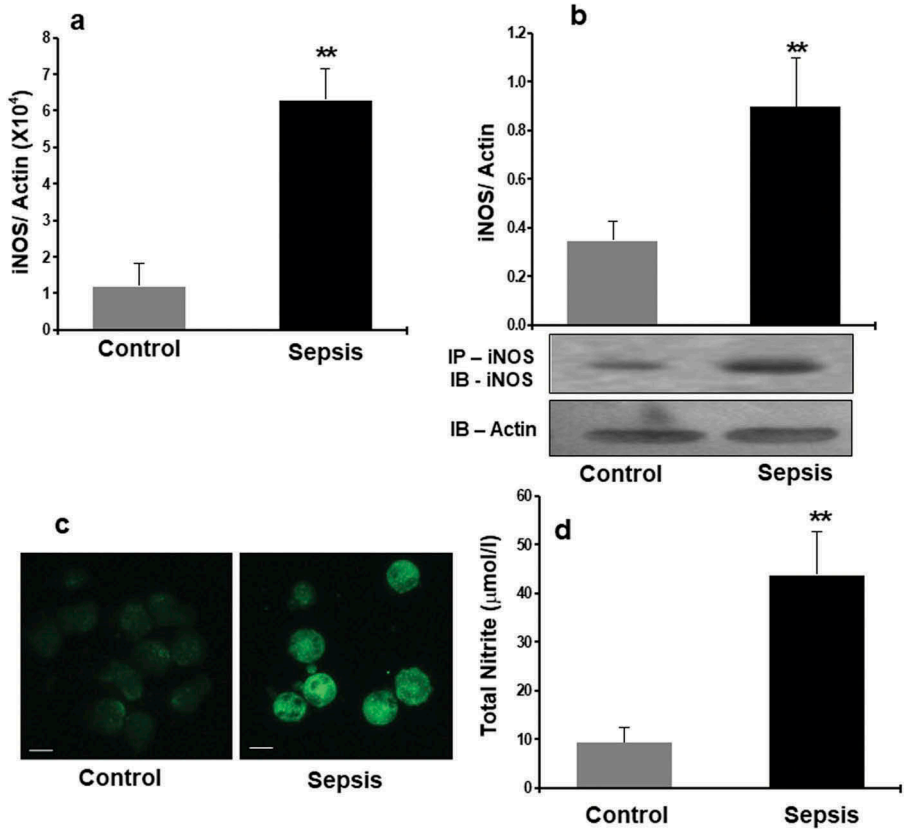

Figure 1. (a) Bar diagrams representing the real-time PCR-based relative mean/SD copy number of iNOS as the ratio of iNOS with housekeeping gene β-actin in control and sepsis neutrophils (**$P < 0.001$). (b) Immunoblot showing the mean/SD level of iNOS protein in control and sepsis neutrophil. β-Actin was used as loading control and to normalise the iNOS protein expression (**$P < 0.01$). (c) Confocal images showing NO generation in DAF-2DA preloaded control and sepsis neutrophils. Images were captured by using ×63 objective lens (bar 10 µm). (d) Bar diagrams representing mean/SD total nitrite content in plasma of control and sepsis (**$P < 0.01$).

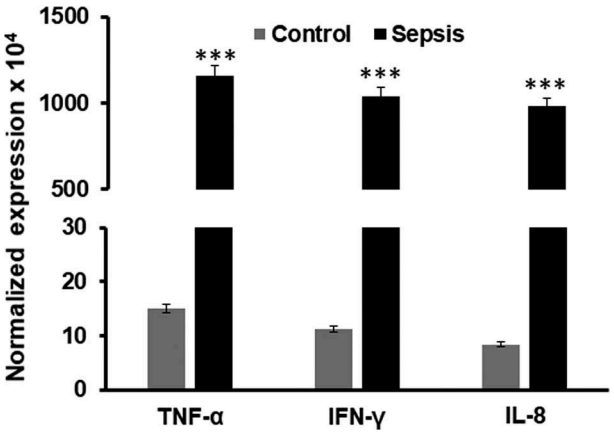

Figure 2. Bar diagrams representing the mean/SD real-time PCR-based relative copy number of cytokines (TNF-α, IFN-γ and IL-8) as the ratio of respective cytokine with housekeeping gene β-actin in control and sepsis neutrophils (all $P < 0.001$).

Table 2. Cytokine correlation analysis in patients with sepsis.

	TNF-α	IFN-γ	IL-8
Total nitrite	0.85	0.64	0.72
SOFA	0.70	0.38	0.57
APACHE II	0.64	0.46	0.57
PaO_2/FiO_2 ratio	−0.64	−0.35	−0.56
Bilirubin	0.58	0.57	0.67
Creatinine clearance	−0.64	−0.43	−0.52

Data are correlation coefficients (r^2). All $P < 0.001$

Our results suggest NO as a potent regulator during infection episodes, perhaps due to the up-regulation of cytokines, which induces iNOS expression and NO generation [7]. It is well known that NO exerts cytotoxic effects when it reacts with the O_2^-, produces $OONO^-$, a potent oxidant, and NO_2 [19]. In aqueous solution, it forms nitrous and nitric acids, thereafter NO_2^-/NO_3^-. The role of these reactive species in the modulation of inflammation and immune regulation is well established.

Nitrite is a stable and inert metabolite of NO that mediates a range of physiological responses in blood and tissue [16,20]. Our results show a significant correlation between nitrite level and SOFA and APACHE II scores, suggesting plasma nitrite could reflect the severity of sepsis as well as the degree of multiple organ dysfunctions [21]. Additionally, cytokine correlations with sepsis severity scores (SOFA and APACHE II) have been reported as suggesting a pro-inflammatory and cytotoxic effect, thereby damaging the tissue at high concentrations [22]. Being excreted from the kidney, excess nitrite could damage the renal endothelium and thus adversely affect the renal

function, as suggested by the inverse correlation with creatinine clearance. In this context, data from a recent human study suggested that nitrite infusion resulted in a vasopressin-independent decrease in CH_2O and urine output. The lack of increase in cGMP accompanying the increase in NO_2 and NOx suggests a direct effect of nitrite or nitrate on the renal tubules and vascular bed, with little or no systemic conversion to NO [23]. Moreover, other studies demonstrated that plasma nitrite level is high in patients with renal failure [24], and nitrite contents are inversely related to systemic vascular resistance, suggesting that NO is an important mediator of increased vasodilatation observed in sepsis [25].

Pro-inflammatory cytokines released by neutrophils and monocytes in sepsis may help in predicting the severity of disease [26]. Several studies on levels of cytokines have established their role as a marker of disease severity and inflammation [27,28], but a major challenge in their use as prognostic marker is their temporally dynamic nature in which there is a rapid shift in cytokine milieu [27,29]. Furthermore, in our study, the correlation between cytokine levels and nitrite could indicate involvement of nitrite in inflammatory response in sepsis [30]. Patients with lower PaO_2/FiO_2 ratios showed higher total nitrite concentration, thus showing that less oxygen from circulating FiO_2 [31] might be related to the overall severity of sepsis, with respect to poor lung function [32]. A correlation between hyperbilirubinemia and outcomes in patients with sepsis has been reported [33], and we note that this marker of liver function correlated with cytokines and total nitrite concentration. Similarly, all laboratory markers correlated inversely with renal function (as marked by creatinine clearance). Creatinine in sepsis is critical and of clinical significance, as severity of sepsis increases in patients with renal insufficiency to some extent perhaps because of the decrease in renal clearance [34,35]. Moreover, several clinical studies have shown that higher serum creatinine clearance is associated with enhanced mortality in acute kidney injury patients [36]. Therefore, in each organ (lung, liver, kidney), levels of the laboratory markers support the hypothesis that they can be used as specific organ disease severity markers, as they appear to do in general markers of disease severity (APACHE II, SOFA).

We acknowledge certain limitations of our study, the principal being that, despite strong correlation evidence, we cannot fully contend that the laboratory markers are causal of the pathology. Nevertheless, this work represents an advance in biomedical science because it provides further evidence of the value of iNOS-derived NO and its products, and inflammatory cytokines, as markers for sepsis severity and organ failure.

Summary table

What is known about this subject?
- NO and its products are neutrophil antimicrobials, but in excessive amounts are cellular cytotoxins *in vitro*.
- Leukocyte-derived cytokines are major inflammatory mediators.
- Sepsis is a clinical state where the immune and inflammatory systems are overwhelmed, leading to organ damage and a high risk of mortality.

What this work adds:
- iNOS-derived NO in neutrophil is increased in sepsis.
- Increased inflammatory cytokines are linked to severity of organ damage in sepsis
- Increased nitrosative stress correlates with organ damage in sepsis

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Disclosure statement

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